Recombinant DNA Technical Bulletin

Volume 2 Number 1
April, 1979

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE Public Health Service National Institutes of Health

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National Institute of General Medical Sciences

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Editors' Note

This is the first issue of the Recombinant DNA Technical Bulletin published following the approval of the 1978 NIH Guidelines on Recombinant DNA Research which became effective January 2, 1979. In light of the significant revisions of the 1976 Guidelines, certain changes have also been made in the Bulletin. In this and future issues, actions which are being considered by NIH and those which have been resolved (such as changes in the Guidelines, new HV2 systems, etc.) will be published, as required by the Guidelines, in the ACTIONS section.

The Bulletin was initiated nearly two years ago by Dr. Michael Goldberg with the purpose of making available to the scientific community current technical information about the newly developing recombinant DNA area. Dr. Goldberg's keen interest and unique abilities were essential in the development of this rather unusual publication. As indicated by the inquiries received at the ORDA office, this information reached and was used by a large number of people. We greatly appreciate Dr. Goldberg's innovative efforts and hard work in developing and publishing the Bulletin.

Now that we are into a new growth phase--particularly regarding new HV systems, new vectors, and the initiation of many experiments which were not feasible under the 1976 Guidelines--we hope to continue to be of service to the scientific as well as the general community. Any comments or suggestions will be appreciated.

Michael A. Resnick, Ph.D.
Editor

Stanley Barban, Ph.D.
Associate Editor

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Introduction

The Recombinant DNA Technical Bulletin is a publication of the Office of Recombinant DNA activities, National Institute of General Medical Sciences. The Bulletin will attempt both to link investigators involved in recombinant DNA research in the U.S. and abroad with the advisory groups and organizations active in this area and to provide prompt notification of certified-host vector systems to a broad community. It will be sent to the chairmen of all institutional biosafety committees registered with ORDA, to all principal investigators of NIH grants and contracts involving recombinant DNA, to the chairmen of all genetic manipulation advisory committees, and to other individuals and groups interestd in recombinant DNA activities.

In accord with its editorial policy, the Bulletin will publish material in the following areas:

1. Scientific information and reports of recent progress in such areas as:
    a.) isolation, purification, and substrate specificity of restriction endonucleases
    b.) development of methods for the construction of recombinant DNA molecules
    c.) development and use of effective and safer cloning vehicles and host cells
    d.) isolation of specific procaryotic and eucaryotic genes and expression of such molecules in different host cells.
    e.) determination and reduction of potential hazards
2. A periodically updated listing of host-vector systems certified by the NIH.
   News and Comments including:
    a.) new and revised versions of national guidelines as issued or proposed by the United States and other countries
    b.) analysis of legislation affecting recombinant DNA research
    c.) reports of scientific meetings
3. Announcements of:
    a.) training courses in experimental and safety aspects of recombinant DNA research
    b.) the availability of contract proposals
    c.) future meetings
    d.) NIH policy issuances concerning recombinant DNA research
    e.) technical information or policy decisions communicated by Institutional Biosafety Committees
4. Bibliography of:
    a.) scientific and technical articles
    b.) articles on public policy, law, and ethics relating to recombinant DNA research.

Contributions in all the above listed areas are welcome, although the Bulletin will continue to emphasize its role as a medium for the rapid communication of items of technical and scientific interest. The Bulletin will have no requirement of annual submission for recipients.

Scientific articles may be submitted in the form of progress notes, abstracts, or summaries of unpublished research findings along with tables, figures and photographs which clarify the text. They may also include discussion and criticism of published reports, technical suggestions, notes of bibliographic omissions, and reports on negative findings. The appearance of such findings in the Bulletin does not constitute publication and should not be referenced as such in the scientific literature. Data reported herein should be considered as tentative and subject to refinement or change prior to publication. Research findings communicated via the Bulletin should be treated as "personal communications" from fellow investigators, and receive credit as such in any situation where questions of priority might arise.

The Bulletin will be published and distributed four times a year at regular intervals. The closing date for receipt of articles will be the first of February, May, August, and November. Communications should be addressed to Editor *Recombinant DNA Technical Bulletin,* Office of Recombinant DNA Activities, Building 31, room 4A52, National Institutes of Health, Bethesda, MD. 20205 USA. This project is funded by the National Institute of General Medical Sciences which, with the exception of articles solicited by the Editor, assumes no responsibility for the content of communications exchanged through the Bulletin.

Instructions to Authors

Contributions should be on plain paper, approximately 8″x10½″ (20x27 cm.) with text width over 7″ (18 cm.). An original and one good copy should be submitted. Pages should be typed single space and in a compact format. Each page of a submission should be sequentially numbered and notation provided as to the location of tables and figures. Authors must assume responsibility for the accuracy of their copy as the Bulletin will not edit unsolicited material.

Communications on research investigations must be short and informal in nature. Unsolicited submissions that constitute or resemble formal manuscripts will not be accepted for publication. In order to facilitate publication, the Editor requests that submitters refrain from the use of subheadings and sections devoted to "Materials and Methods," "Summary," "Acknowledgements," and "Bibliography." References, when appropriate, should be incorporated into the text in short form.

Scientific Reports

The Scientific Reports section of this and the next issue is devoted to host-vector systems involving hosts other than E. coli. *In this issue there are reports on various* Bacillus *species and possible vectors. In addition the next issue will include reports on HV1 and HV2 systems in the yeast* Saccharomyces cerevisiae. *At the time of this publication, there are no approved HV1 systems;however, HV1 and HV2 lower eukaryote and HV1* Bacillus subtilis *systems are presently being evaluated by NIH (see Issues Pending).*

Pathogenic Bacteria of Pest Insects as Host-Vector Systems

Robert M. Faust, Ph.D.
United States Department of Agriculture
SEA, AR, NER
Plant Protection Institute
Insect Pathology Laboratory
Agricultural Research Center
Beltsville, Maryland 20705

In the formulation of the new revised guidelines published in the *Federal Register* (December 22,1978) attention was focused on bacteria which have the potential for pathogenicity to economic plants and animals. There are, of course, bacteria which are beneficial to man from an environmental viewpoint. Among the "beneficial" bacteria are those which are pathogenic to specific pest insect species. Twenty years of research by scientists in the USDA, industry, and at the university level have concentrated on establishing the safety of some of these bacteria with regard to plants, vertebrates and non-target invertebrates. The safety tests were sufficiently convincing to the U.S. Environmental Protection Agency (EPA) to permit their use as biological insecticides in both agricultural, community, and forest pest-management systems.

Considering the importance these bacteria have assumed in terms of environmental improvement (i.e. by reducing dependence on non-specific chemical insecticides), the safety of these bacteria with regard to non-target organisms, and the desirability of genetic engineering research to develop or more fully understand these biological insect control agents, this material is presented here to inform interested researchers and the Recombinant Advisory Committee on the nature of these bacteria, document the safety of these bacteria as possible model host-vector systems for a variety of recombinant DNA studies and give rationale for their certification as hosts for HV-1 systems.

1. *The Nature of Entomopathogenic Bacteria*

The "beneficial" insect bacterial pathogens may be defined as those bacteria which are pathogenic to specific pest insects. Included in this group are the more than 20 varieties of *Bacillus thuringiensis, B. popilliae, B. lentimorbus, B. euloomarahae, B. fribourgensis, B. sphaericus, B. moritai,* certain strains of *B. cereus, Clostridium brevifaciens, and C. malacosomae.* The first eight of these organisms are presently being used commercially or show the greatest potential for use.

Other entomopathogenic bacterial species have been found in *Pseudomonas*, *Aerobacter* spp., *Cloaca* spp. and *Proteus*, but have not been seriously considered for use in biological control of pest insects owing to their non-specificity.

A. Mode of action and host range.

All entomopathogenic spore-forming bacteria produce endospores which allow them to persist in a dormant or quiescent state outside the intended host. Upon ingestion by a susceptible host the spores may germinate in the gut. In obligate pathogens in the genus *Bacillus*, the vegetative cells produced by the germinating spores enter the hemocoel where they multiply rapidly, destroy certain tissues, and soon fill much of the cavity. Prior to death of the host, thick-walled refractile spores are formed which appear white through the integument, thus the name "milky disease". The causative organisms of "milky disease" are *B. popilliae*, *B. lentimorbus*, *B. fribourgensis*, and *B. euloomarahae*, and affect primarily beetle larvae of the insect order Coleoptera. Following death, the host disintegrates and the spores are released into the soil. The mode of action of obligate pathogens of the genus *Clostridia* differs in that these bacteria multiply only in the gut and do not invade the hemocoel.

The crystalliferous sporeformers (varieties of *B. thuringiensis)*, in addition to forming endospores, produce a proteinaceous parasporal crystal in the sporangium at the time of sporulation. The crystal contains an endotoxin capable of paralyzing the gut of most pest lepidopterous larvae (Heimpel, 1967) and some pest mosquito larvae depending on the *B. thuringiensis* strain (de Barjac, 1978a, b). This material has been labeled as δ-endotoxin (Heimpel, 1967) and susceptible insects are killed by the toxic crystals (Heimpel and Angus, 1963). In addition to its insecticidal properties, the intracellular proteinaceous crystal produced by *B. thuringiensis* apparently is capable of regressing tumors (Prasad and Shethna, 1975). In addition to the crystal, at least three other substances toxic to insects may be produced by various strains of crystalliferous bacteria. Following the nomenclature proposed by Heimpel (1967) these are: α-exotoxin, an enzyme of the growing bacterium; phospholipase C, that breaks down essential phospholipids in ensect tissue; β-exotoxin, a small, water-soluble, heat-stable nucleotide which is a structural analogue of ATP that inhibits DNA-dependent RNA polymerase (Sebesta and Horska, 1968) and kills larvae and pupae of Diptera and some Lepidoptera; and γ-exotoxin, an unidentified phospholipase that affects phospholipids, probably releasing fatty acids from the molecule. The β-exotoxin has been demonstrated to show toxicity to mammals, however, a number of varieties of *B. thuringiensis* do not produce the β-exotoxin (Faust, 1975) and these varieties are being used in commercial formulations.

B. cereus, a spore-forming facultative pathogen, also produces phospholipase C in quantities sufficient to damage gut cells of susceptible species, thus aiding entry of the bacterium into the hemocoel (Heimpel 1955; Heimpel and Angus, 1963). The non-spore-forming entomopathogenic bacteria are a heterogeneous group that usually infect insects only under extraordinary circumstances. Although they occur in the gut of the host, they do not multiply readily there and probably do not produce enzymes or toxins in sufficient quantities to cause damage or to aid invasion of the hemocoel. They do, however, multiply in the hemocoel of the insect once they have invaded it (Bucher, 1963).

Clostridial pathogens were originally isolated from *Malacosoma californicum (pluviale)* (Bucher, 1957). Other than experimental infections in other tent caterpillars and the possible presence of these bacteria in *Thymelicus lineola* (Heimpel and Angus, 1963) little is known about their host range. *B. thuringiensis* and its varieties have been tested successfully against more than 137 insect species from the orders Lepidoptera, Hymenoptera, Diptera, and Coleoptera (Heimpel, 1967). Most of these pathogenicity tests have been conducted in the laboratory. The most susceptible insects are those lepidopterous larvae having alkaline gut contents (pH 9.0-10.5) and enzymes which dissolve the crystals and release the toxin (Angus, 1956; Angus and Heimpel, 1959; Heimpel and Angus, 1959).

B. cereus is a common, widely distributed soil saprophyte with an extensive host range including insect species from the orders Coleoptera, Hymenoptera and Lepidoptera (Heimpel and Angus, 1963). Its host range is also limited by the pH of the gut as a highly alkaline gut content can inactivate the toxic exoenzyme, phospholipase C (Heimpel, 1955).

The non-spore-forming bacteria attack a wide range of hosts from the Acarina, and the insect orders Coleoptera, Diptera, Hymenoptera, Isoptera, Lepidoptera, and Orthoptera (Bucher, 1963).

B. Methods of propagation and factors which influence effectiveness.

The obligate pathogens, among species of Bacillus and Clostridium, are fastidious organisms that can be propagated in artificial media only with extreme difficulty. The commercial preparations of *B. popilliae* produced in the U.S.A. are made by injecting or feeding bacteria to grubs of the Japanese beetle. The bacteria multiply to great numbers in the grubs and are recovered by grinding up the host.

Most other bacterial pathogens are much less fastidious and can be readily propagated on simple bacteriological media. This property is one reason why the facultative bacterial pathogens are likely candidates for microbial control.

Unfortunately, there are several obstacles to the commercial production and wide-spread use of many biological control agents in lieu of many of the well-known and hazardous chemical insecticides. For example, the wide-scale biological control of the Japanese beetle, *Popilla japonica* Newman, the European chafer, *Amphimallon majalis* Razoumowsky and other susceptible scarabaeid grubs, major pests of lawns, pastures and other plant life in many parts of the world, could be considerably facilitated by the development of an *in vitro* industrial method for spore production of *B. popilliae*, the causative agent of milky disease in these insects. Spore preparations of *B. popilliae* are produced commercially by collecting living larvae from infested soil, injecting each grub with the disease organism, incubating the larvae until the blood becomes filled with spores and then grinding and mixing them with an extending material such as talc. This procedure results in an expensive low-yielding product incapable of meeting the requirements for adequate mass control of these serious insect pests throughout the world.

Another organism, *Bacillus sphaericus*, a potentially powerful biological control agent for mosquitoes, is not readily grown in cheap culture media, but requires rather expensive nutrient additives for optimum production. Under our present knowledge and technology, this organism would not be commercially feasible for industry to develop at this time and hence its widespread use is hindered in lieu of such organophosphate insecticides as malathion. The pathogenicity of this organism for mosquito larvae seems to reside in the capacity for the production of an endotoxin specific for its host midgut (Davidson et al., 1975). Recombinant DNA incorporation of the genes responsible for production of this toxin and transformation into *B. thuringiensis*, the biological lepidopteran insecticide being produced at a price competitive with chemical insecticides, could yield a broader-spectrum biological control agent affecting several orders of economically important insect pests and although broader based, this new strain would be selective, an attribute not contained in many chemical insecticides. Their development and use could result in a decreased use of hard chemical insecticides. In fact, a recently isolated strain of *B. thuringiensis* (var. *israelensis)* has demonstrated that the parasporal crystals (δ-endotoxin) is toxic for such pest mosquito larvae as *Aedes aegypti* and *Anopheles stephensi* but is not toxic to larvae of Lepidoptera (de Barjac, 1978a). Combining of these pathogenic entities into one strain would give commercial industry a very valuable and fruitful product.

2. General Safety Considerations Pertinent for Approval as HV-1 Hosts

One of the first entomopathogenic bacteria to be used extensively in the field was the milky disease organism, *Bacillus popilliae*, isolated and described by Dutky (1940) and has been used on the Eastern seaboard since the early 1940's to control the Japanese beetle, *Popillia japonica*. The sole producers of this bacterial product is the Fairfax Biological Laboratory at Clinton Corners, N.Y. and Reuter Laboratories, Inc. at Haymarket, Virginia. The materials produced by Fairfax Biological Laboratory is manufactured under the trade names of "Japanese beetle Doom" (USDA Reg. No. 403-9) and "Japidemic" (USDA Reg. No. 403-14). Reuter Laboratories manufacture their product under the trade name of "Milky Spore" (EPA Reg. No. 36488-1). The results of published and unpublished tests demonstrated that the milky disease bacteria are innocuous to turf, invertebrates and vertebrates, including man (Heimpel, 1971; Bailey, 1971; Heimpel and Hrubant, 1973; Bulla *et al.*, 1975; Anonymous, 1977; Ignoffo, 1973). The protocols for these tests were informally discussed with the Environmental Protection Agency and the United States Department of Agriculture personnel, and the tests as reported were conducted by qualified investigators in accordance with protocols found acceptable to and recommended by officials of these agencies. The following tests were conducted to obtain information on the safety of *B. popilliae* to man and other mammals: (1) Safety evaluation by repeated oral administration of *B. popilliae* to rats and monkeys; (2) Clinical examination of production personnel exposed to *B. popilliae;* (3) Medical and serological examinations of humans engaged in production of *B. popilliae;* (4) Microbiological examination of commercial preparations of *B. popilliae;* (5) Published and unpublished studies demonstrating its specificity to beetle larvae and safety to vertebrates, including humans; (6) Acute dermal toxicity in guinea pigs of a spore preparation of *B. popilliae;* (7) Acute eye irritation potential study in rabbits of a spore preparation of *B. popilliae;* and (8) inability of *B. popilliae* to grow at mammalian or avian body temperatures. The data supported the relief sought by the petition, namely, exemption from the requirement of a tolerance. *B. popilliae* is so specific that its use, or even its misuse, cannot result in any threat to the safety of humans, or other animals. It has been shown by the data obtained to be completely innocuous wherever used and at whatever levels. The milky disease organism has never been shown to infect anything other than Japanese beetles and certain closely related beetles. Application of up to 2000 pounds per acre of milky disease spore powder to pastures caused no discernible harm to the turf. The effect of temperature is a line of defense of vertebrates against infection by milky disease bacteria. The maximum temperature for multiplication of *B. popilliae* is below the minimum body temperature of domestic vertebrates. The organism has been shown not to germinate in the digestive tract or persist in the feces of vertebrates in these tests.

Bacillus thuringiensis has been commercially available in the United States since 1958 and is widely used to control caterpillar pests of food crops, fiber crops and forests. Both laboratory and commercial preparations of *B. thuringiensis* have been evaluated for specificity and possible toxicity-pathogenicity to vertebrates since 1957. International Minerals and Chemical Corp. (IMC) produced the first commercial preparations of *B. thuringiensis* (Thuricide R) in July 1957. Toxicology of Thuricide was included in a petition to the Food and Drug Administration (FDA) that resulted in a temporary exemption from tolerance in December 1958 and a full exemption from tolerance for use on food and forage crops in April 1960. The Canadian Department of Agriculture granted a similar registration for Thuricide in November 1961. Another petition, that contained safety evaluation data, was submitted by Nutrile Products, Inc. (NPI) for their preparation of *B. thuringiensis* (Biotrol-BTB R) in June 1959. The petition on Biotrol-BTB presented results on the following studies: intraperitoneal injection in the mouse, guinea pig, rabbit, swine, and chick; serial blood passage after intraperitoneal injection into mice; and ingestion by the rat, chick and human. Subsequent studies by NPI included subacute feeding tests in birds and mammals. Another commercial preparation of *B. thuringiensis* (var. *kurstaki,* Dipel R), produced by Abbott Laboratories, was recently developed and registered in 1970 (EPA registration No. 275-18-AA). Preparations were evaluated for their safety to fishes, birds, and mammals, including man. Initial studies were designed to establish that *B. thuringiensis* or its varieties were neither pathogenic, allergenic, nor able to persist in mammals or to answer questions concerning the possibility that *B. thuringiensis* may mutate or be selected for pathogenicity to humans. Acute and subacute studies were carried out to establish requirements for registration of commercial preparations of *B. thuringiensis.*

The results (Anonymous, 1978) demonstrated that *B. thuringiensis* has no known adverse effect on man, pets, birds, fish, earthworms, beneficial insects or plants. Other studies revealed no evident differences in density of snails, *Forficula,* myriapodes, and wood lice after treatment (Benz and Altwegg, 1975). Millions of pounds of Dipel have been used without a single human toxicity or environmental damage report. Repeated tests have shown that *B. thuringiensis* does not inhibit plant growth and was non-phytotoxic after testing for phytotoxicity on more than 140 species of plants. No evidence of acute or chronic toxicity in rats, guinea pigs, mice, swine, humans or other mammalian test animals has been found. *B. thuringiensis,* either in the form of the commercial product (Dipel) or as naturally occuring *B. thuringiensis* has no harmful effect on the environment for several reasons, all of which are characteristic of the bacteria (Anonymous, 1978; Forsberg *et al.,* 1976): (1) Natural *B. thuringiensis* numbers in the soil are limited by its metabolic requirements; it is apparently unable to compete effectively with other soil bacteria and unlike *B. cereus, B. thuringiensis* is seldom isolated and reported from soil but is found and isolated from disease larvae. Cadavers of insects killed by *B. thuringiensis* contain vegetative cells but few spores and crystals, the principal agents of mortality rarely build up in the insect population. (2) Conditions on the surfaces of living plants are not suitable for substantial growth of *B. thuringiensis,* (3) Numbers of *B. thuringiensis* organisms and spores in the environment are reduced quickly, even after applying concentrated commercial formulations, by ultraviolet light, i.e., the number of recoverable *B. thuringiensis* spores usually falls to background levels of natural *Bacillus* populations in 2 or 3 days, (4). *B. thuringiensis* is toxic to target insects only when ingested, and (5) there is little prolific multiplication of *B. thuringiensis* in insect larvae and when it does occur it takes place just before or after death of the larvae (Prasertphon *et al.,* 1973). The organism has been proven harmless to vertebrates and invertebrates in terrestial, marine and fresh water environments even when test dose levels of 1000 to 2000 times the normal use rate were used. Laboratory studies indicated that ingested *B. thuringiensis* is eliminated from test animals within hours after ingestion (Anonymous, 1978). Other studies have demonstrated vegetative cell counts of *B. thuringiensis* decreased by 90 percent within 4 hours when bacterial suspensions were introduced into the rumens of cattle with commercial spore preparations decreasing after 24 hours (Adams and Hartman, 1965). No spore germination of spores in the rument were noted in that study. Studies of the survival of *B. thuringiensis* in the digestive tracts and feces of mammals and birds showed similar results although spores survived passage (Smirnoff and MacLeod, 1961).

Extensive experimental work on safety of *B. thuringiensis* (and certain strains of *B. cereus*) has also been reported by Fisher and Rosner (1959), Ignoffo (1973), Heimpel (1971), Krieg and Franz (1959), Lemoigne (1956), Steinhaus (1951), and Lamanna and Jones (1963) and include virulence in mice, persistence in blood of mammals, pathogenicity by parenteral administration, inhalation toxicity in mice, allergenicity in guinea pigs, inhalation and ingestion by human volunteers and acute oral toxicity.

Bacillus moritai, a pathogen for houseflies and mosquitoes, has been manufactured by private industry in Japan and investigations have been made on the effectiveness of its product in the field and the safety for warm-blooded animals (Burgerjon, 1973; Fujiyoshi, 1973). *B. moritai* is not pathogenic for the silkworm, honeybee, mouse, rat, rabbit, bird, fish, pig, or cattle. Malignancy and carcinogenicity were also examined and finally the safety was checked by human experiments with negative results.

3. Features Relating to Recombinant DNA Experimentation

Entomopathogenic bacteria of pest insect species offer a unique and relatively safe model system for studying the effects of recombinant DNA research using procaryotic - and possibly eucaryotic - derived DNA. The entomopathogenic bacteria are primarily species or genus "specific" and several have been tested for their safety in regard to vertebrate and non-target organisms. It is suggested that increased recombinant DNA research emphasis be focused on these bacteria which have some safety advantages over some other bacteria currently used as recombinant DNA tools. Envisaged in research among entomopathogenic bacteria are possible applications of *in vitro* gene splicing and recombinant DNA cloning to (1) expand the insect pest host-spectrum of existing insect pathogens, (2) develop new and more potent strains of pest insect pathogens, (3) improve the physiological tolerance and epidemiological properties of these bacteria, and (4) facilitate the *in vitro* commercial production of the more fastidious insect pathogens by expanding the range of *in vitro* substrates upon which they can grow.

The use and approval of appropriate plasmid or bacteriophage vectors is a primary consideration in this research. Plasmids have been identified in many bacteria (Cohen, 1976; Helenski and Clewell, 1971; Helinski, 1973). A large variety of specific biochemical functions such as fertility, resistance to antimicrobial drugs, production of bacteriocins, production of toxins, etc., have been attributed to these genetic elements. We have recently examined four entomopathogenic bacteria for extrachromosomal DNA molecules which are summarized in Table 1.

Table 1

Number and size estimation of extrachromosomal DNA elements of *Bacillus thuringiensis* var. *kurstaki*, var. *sotto*, var. *finitmus*, and *Bacillus popilliae* isolated by agarose gel electrophoresis.

B. t. var *kurstaki*	*B. t.* var *sotto*	*B. t.* var *finitimus*	*B. popilliae*
$>50 \times 10^6$ [a]		$>50 \times 10^6$(2)	
~45 x 10^6			
~29.9 x 10^6	~23.5 x 10^6		
~17.1 x 10^6			
7.4 x 10^6			
4.2 x 10^6			
3.9 x 10^6			4.45 x 10^6
3.6 x 10^6			
1.1 x 10^6		0.98 x 10^6	
0.87 x 10^6			
0.80 x 10^6	0.80 x 10^6	0.79 x 10^6	
0.74 x 10^6	0.62 x 10^6		0.58 x 10^6

a/ Daltons; size estimations were determined from a standard curve (full log; 3 cycles by 1 cycle) estimated by their mobilities relative (R_f) to the DNA standards included in the agarose gels.

The basic characteristics of the larger isolated elements, especially the giant DNA elements, are not unlike those of representative plasmids isolated from members of other genera of bacteria (Clowes, 1972). However, assignment of specific genetic functions to any of the *B. thuringiensis* or *B. popilliae* DNA elements is speculative at this time. There is some evidence, however, indicating that plasmid(s) may be involved in the synthesis of the parasporal crystals (*B.t. δ-endotoxin*) that are responsible for the pathogenicity of *B. thuringiensis* to pest insects (Debavov *et al.*, 1977; Stahly *et al.*, 1978).

Most of the described plasmids in bacilli (Lovett and Bramucci, 1975; Lovett *et al.*, 1976; Tanaka *et al.*, 1977) are crytic elements lacking genetic markers and are unsuitable for selection of transformed colonies. Bernhard *et al.* (1978), however, have undertaken a search for plasmids in *Bacillus* species, mainly *Bacillus cereus* and *B. subtilis,* to characterize their properties and develop their potential use as vectors for gene cloning. Most of the *B. cereus* strains contained two or more plasmids with molecular weights ranging from 1.6 x 10^6 to 105 x 10^6. Bacteriocin production could be attributed to a 45 x 10^6-dalton plasmid from *B. cereus,* and tetracycline resistance to a 2.8 x 10^6 plasmid from *B. cereus.* The plasmid carrying resistance to tetracycline which was originally isolated from *B. cereus,* could be subsequently transformed in *B.subtilis,* where it was stably maintained. Varietal types of *B. thuringiensis* have been shown to be resistant to streptomycin (Afrikian, 1960), penicillin, polymyxin B, nystatin, Bacitracin, viomycin (Ignoffo, 1963; Krieg, 1969), oxytetracycline (Fargette and Grelet, 1976), and tetracycline (Fargette *et al.*, 1978; Rapport *et al.*, 1978). Bacteriocin production has also been identified in *B. thuringienses* (Krieg, 1970). Obviously, further genetic and biochemical studies are necessary for the determination of the biological functions of extrachromosomal DNA elements in *B. thuringiensis* and *B. popilliae* and to definitely determine which isolated elements are indeed autonomous replicons.

Asporogenic mutants of *B. thuringiensis* are available (Nishiitsutsuji-Uwo and Yoshiharu, 1975; Yousten, 1978) to preclude the supposed problem of persistence through sporulation. However, if the researcher is not combining potentially hazardous DNA or hazardous heterlogous markers, or in cases where *B. thuringiensis, B. popilliae,* or other safe entomopathogenic species, is being amplified in the same or other entomopathogenic species shown safe, I do not see the necessity of restriction to asporogenous strains.

A number of bacteriophages have been reported in entomopathogenic bacteria and some have been suggested for use in mediating generalized transduction in *B. thuringiensis*(Thorne, 1978; Van Tassell and Yousten, 1976; Ackermannn et al., 1974; Chapman and Norris, 1966; Colasito and Rogoff, 1969; De Barjac et al. 1974; Norris, 1961; Yoder and Nelson 1960). In fact some examples of cotransduction of linked markers in *B. thuringiensis* have been presented, demonstrating the feasibility of chromosomal mapping in this organism (Thorne, 1978). Two linkage groups were demonstrated. One group included linkage of trp-1 to cys-1 and cys-2 but not to met-1. The second group included linkage of met-1 to arg-1 and arg-2 but not to arg-3. The cys-1 and cys-2 mutants were able to grow on cysteine, methionine, homocysteine, or cystathionine, but not on sulfide. Mutations conferring this phenotype are not represented on the current *B. subtilis* chromosomal map (Young and Wilson, 1975). The met-1 mutant has a strict requirement for methionine and may be analogous to metC or metD of *B. subtilis.* The arg-1 and arg-2 mutants were able to grow on arginine, ornithine, or citrulline and may be analogous to arg0 mutants of *B. subtilis,* whereas the arg-3 mutant which grew only on arginine may be analogous to argA. Another bacteriophage (CP-51) grown in eight varieties of *B. thuringiensis* has similar characteristics to those of the phage grown in *B. cereus.* Since this phage successfully transduced several auxotrophic markers in *B. cereus,* it appears possible that it will also be able to mediate gene transfer in *B. thuringiensis* (Van Tassell and Yousten, 1976). Transduction studies involving transducing phages and auxotrophic mutants would probably contribute significantly to an understanding of the molecular biology of *B. thuringiensis* and to subsequent genetic engineering for producing more virulent strains.

There are some disadvantages of the entomopathogenic bacteria for use as host systems. Chromosomal mapping and the knowledge of genetics and physiology of plasmids and bacteriophages is ill-defined in these bacteria. High frequency, specialized transformation and transduction is not well known as a means of gene enrichment. To my knowledge the only work described on transformation in *B. thuringiensis* is that of Reeves (1966). Transformation of DNA mediating the production of *β*-exotoxin and *δ*-endotoxin from *B. thuringiensis* var *thuringiensis* to *B. thuringiensis* var *finitimus* was described. *B. thuringiensis* var *finitimus* does not produce *β*-exotoxin, and the parasporal crystals produced by this variety are not toxic to most Lepidopteran larvae, especially the cabbage looper and salt marsh caterpillar. Transformed isolates of *B. thuringiensis* var *finitimus,* which can be distinguished from other varieties of *B. thuringiensis* by colony and crystal spore morphology, were found to produce β-exotoxin and parasporal crystals toxic to the cabbage looper.

This laboratory is currently studying the transformation system in *B. thuringiensis* var *kurstaki* and *B. popilliae* using the pUB 110 from *Staphylococcus aureus* that mediates kanamycin/neomycin resistance (Lacey and Chopra, 1974).

In considering selected entomopathogenic bacteria as hosts for HV-1 systems it is well to keep in mind that under the new revised guidelines new HV1's need not offer a distinct advantage over *E. coli* K-12 host-vectors, need not be capable of modification to HV2 and HV3, and need not be class 1 etiologic agents. *B. thuringiensis* and *B. popilliae* enjoy EPA registration and are known to be harmless to humans, domestic animals, wildlife, plants, honeybees, and other non-target organisms. Neither *B. thuringiensis* nor *B. popilliae* established themselves in the normal bowel or multiply in the alimentary tract, although spores remain viable during passage through the intestinal tract. Although *B. popilliae* and *B. thuringiensis* survive only in the spore stage in the environment, they only affect specific target pest insects and *B. thuringiensis* normally does not multiply in the target insect. Neither are capable of spreading from animal to animal or plant to plant except target insects. They do not multiply on body surfaces or intestines and lungs nor penetrate animal cells or spread through animal bodies except target pest insects. They only produce toxins that affect specific pest insects (with the exception of the few varieties of *B. thuringiensis* producing the β-exotoxin which are not approved for use by EPA), but otherwise do not alter other living things to cause disease. They do not resist normal body defense mechanisms, or establish themselves as permanent residents of human beings or other non-target organisms.

It would, therefore, appear that these two organisms meet the requirements for use as host in HV-1 systems. I propose that the *Staphylococcus aureus* plasmids, which have already been approved for cloning DNA in *Bacillus subtilis,* could be used in *B. thuringiensis* and *B. popilliae* under the same containment conditions. Shotgun experiments with DNA's from either *B. thuringiensis* or *B. popilliae* using plasmid vectors from *Stathylococcus aureus* or plasmid vectors from, for example, *B. thuringiensis* into *B. popilliae,* or vice versa, would be performed under P2 containment. Likewise, DNA's from other *specific* insect pathogens meeting the safety criteria for registration, such as *Bacillus sphaericus, B. moritai,* or recombinant DNA entomopathogenic bacterial phages would be performed under P2 physical containment. Other recombinant DNA experiments using *B. thuringiensis, B. popilliae* and/or entomopathogenic bacteria as hosts that meet the safety criteria for registration would be performed under the physical containment requirements specified in the guidelines for HV-1 systems. Varieties of *B. thuringiensis* producing the β-exotoxin would not be allowed for use under these containment conditions.

It is suggested that increased recombinant DNA research emphasis be focused on these bacteria which have some safety advantages and possible agricultural benefit over other bacteria currently used as recombinant DNA tools and that the entomopathogenic bacteria deemed safe by the Environmental Protection Agency as biological insecticides be considered for approval as hosts for HV-1 systems. Lastly, it is unlikely that agriculture will best be served through the use of *E. coli* K-12 or *B. subtilis;* alternate host-vector systems are essential if the potential of recombinant DNA technology for agriculture is to be realized.

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ϕdo Phages: Helper Independent Temperate Phage Molecular Cloning Vehicles for *Bacillus subtilis*

D.H. Dean[1,2,3,4], J.B. Perkins[1], J.M. Kroyer[1]
M.S. Rudinski[1], I.B. Lazdins[1], P.A. Martin[2],
W.F. Martin[3], and F.R. Perro, Jr.[2],
Departments of Microbiology[1], Genetics[2],
Development Biology[3], and the *Bacillus* Genetic Stock Center[4]
The Ohio State University, Columbus, Ohio 43210

Until very recently the use of recombinant DNA techniques has been almost exclusively limited to *Escherichia coli.* Recently, however, plasmid cloning vehicles have been introduced from *B. subtilis* (1,2,3,4,5). The value of DNA cloning to the group of bacteria related to *Bacillus subtilis* is readily apparent when one considers the wealth of their genetic and economic capabilities. More than eighty antibiotics and a dozen enzymes of economic importance are made by *B. subtilis, B. amloliquefaciens, B. licheniformis* or *B. pumilus* (6,7). Of particular value is the ability of these organisms to excrete certain of these enzymes obviating the necessity to lyse cells to harvest the enzymes. *Bacillus* also has the ability to form endospores and this genetically complex developmental process, involving thirty to forty genes, represents a complex procaryotic developmental system. Other genetic capabilities include generalized transduction, transformation and a new plasmid transfer technique (S. Chang and S. Cohen, Mol. Gen. Genet., *in press*). The expected value of recombinant DNA technology to *Bacillus* research is to amplify genetic products, provide new approaches to the study of sporulation and to allow *B. subtilis* to assume the role of a choice host for a wider range of genes coding for industrially important products.

Several years ago we set out to develop phage molecular cloning vehicles similar to the lambda systems available for *E. coli* (8,9,10,11). This task has not been as easily achieved as for lambda because the information about *Bacillus* phages was scanty in comparison. The approach to developing a phage cloning system for *B. subtilis* has proceeded through the following steps:

1. *Isolation and Characterization of New Temperate subtilis Phages.*
This was necessary since few temperate subtilis phages were previously known. We concentrated on temperate phages to take advantage of their powers of integration in order to develop a transduction system capable of achieving low copy alleleic complementation. These studies have resulted in a growing understanding of temperate phages of *Bacillus* (12,13,14,15) including one of the few examples of the comparison of a group of phages by classical criteria (12), restriction enzyme analysis (13) and heteroduplex analysis (M.S. Rudinski and D.H. Dean, manuscript submitted). These studies have shown that the temperate *subtilis* phages separate into at least four groups (Table 1, (12)) which is surprising, given the fact that most of the hosts listed in Table 2 are very closely related and exchangers of DNA with the transformable strain 168. This narrow host range might be a useful attribute for certain cloning procedures where promiscuous interspecies transfer of cloned DNA would be undesirable.

2. *Restriction Enzyme Analysis of Temperate Phages.* Our goals were to analyze new phages with restriction enzymes which generate cohesive ends and to identify that phage which has one (or a few) restriction sites. We did not pursue the group III phages, o3T and p11 (13) because of their large genome size and the numerous restriction sites with all enzymes examined. The group I phages were appealing in having a smaller genome size (all about 25 x 10^6 daltons). Extensive enzyme mapping has been done (14,15, *Microbial Genetics Bulletin* 43: 7-10 (1977)). It is apparent from Fig. 1 that p14 has a single *Sal*GI site and a single *Bgl* II site. The close relationship between p14 and ϕ105 (13,14,15, 83% homoduplex, Rudinski and Dean unpublished) has lead us to speculate (14,15, *Microbial Genetics Bulletin op cit*) that the *Sal*GI site of p14 is in the p14 immunity region. Although the *Sal*GI site is extremely close to the immunity region we have yet to remove it by a series of clear plaque deletions into this region. Furthermore, cloning into the *Sal*GI site does not result in clear plaques (it does however appear to make the plaque appear discerningly less turbid than normal).

3. *Isolation of Deletion Mutants.* In order to clone larger fragments, deletion mutants of ρ14 have been obtained by sodium pyrophosphate selection. Turbid plaque deletion mutants, called ϕdo, are exemplified by odo7 a 1.9 x 10^6 dalton deletion in the *E*coRI A fragment (see map Fig. 1). Clear plaque deletion mutants, called ϕdoc are available with 1-1.5 x 10^6 dalton deletions with a predicted maximum of 5 x 10^6 in the *E*coRI A and F bands (see map Fig. 1) (J.M. Kroyer, J.B. Perkins,

M.S. Rudinski, and D.H. Dean, in preparation). Fragment band shifts between p14 and odo7 are shown in Fig. 2. These cloning vehicles provide the mechanism for integrating cloned DNA by insertion into the host chromosome (to achieve low copy allelic complementation), or lytic cycle amplification/expression of cloned DNA during the lytic cycle.

4. *Demonstration of Cloning.* According to present indications, ϕdo7 vehicles carrying cloned DNA make a discernably lighter turbid plaque than ϕdo7. Both agarose gel restriction analysis and heteroduplex analysis of phage from such plaques, called *x* phages, reveal cloned DNA is carried. *E*coRI analysis of *x*5 (Fig. 2) shows an absence of the F band and appearance of a new higher molecular weight band. Since the *Sal*GI site is within the *E*coRI F band, this result is consistent with extra DNA inserted into the *Sal*GI site. Fig. 3 (data of J.B. Perkins, J.M. Kroyer, and D.H. Dean, manuscript in preparation) shows heteroduplex molecules of *x*5 and p14. Arrows indicate the inserted DNA (R-DNA) and the ϕdo7 deletion loop(Δ). *x* 5 carries 0.5 x 10^6 dalton of identified cloned DNA. Current work involves removal of all but one *E*coRI site (the one joining fragment A and F) which would allow cloning with *E*coRI and the insertion of an adaptor molecule for *Bam*HI into the *Sal*GI or *E*coRI site.

This phage cloning vehicle in conjunction with the currently available plasmid cloning vehicles (1,2,3,4,5) will provide Recombinant DNA systems for further genetic studies of *B. subtilis.*

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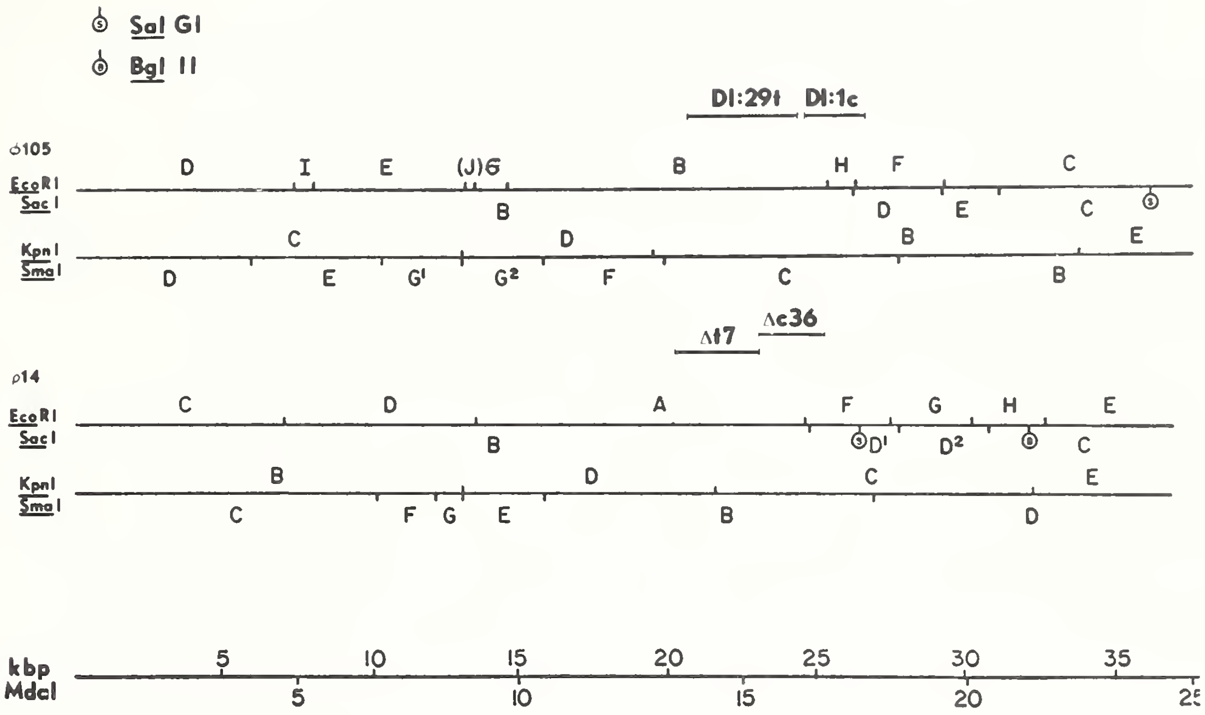
Figure I.



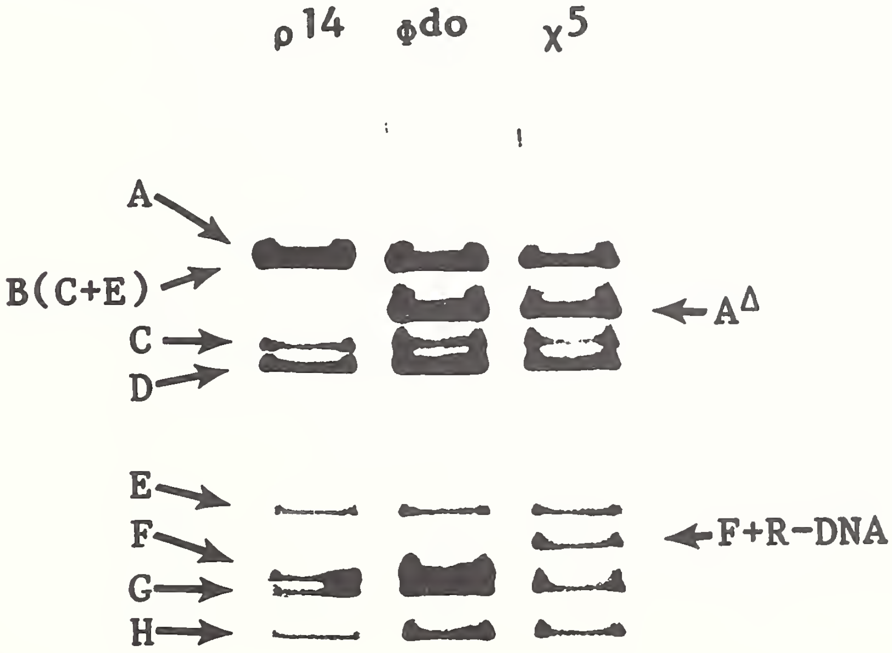


Figure II.

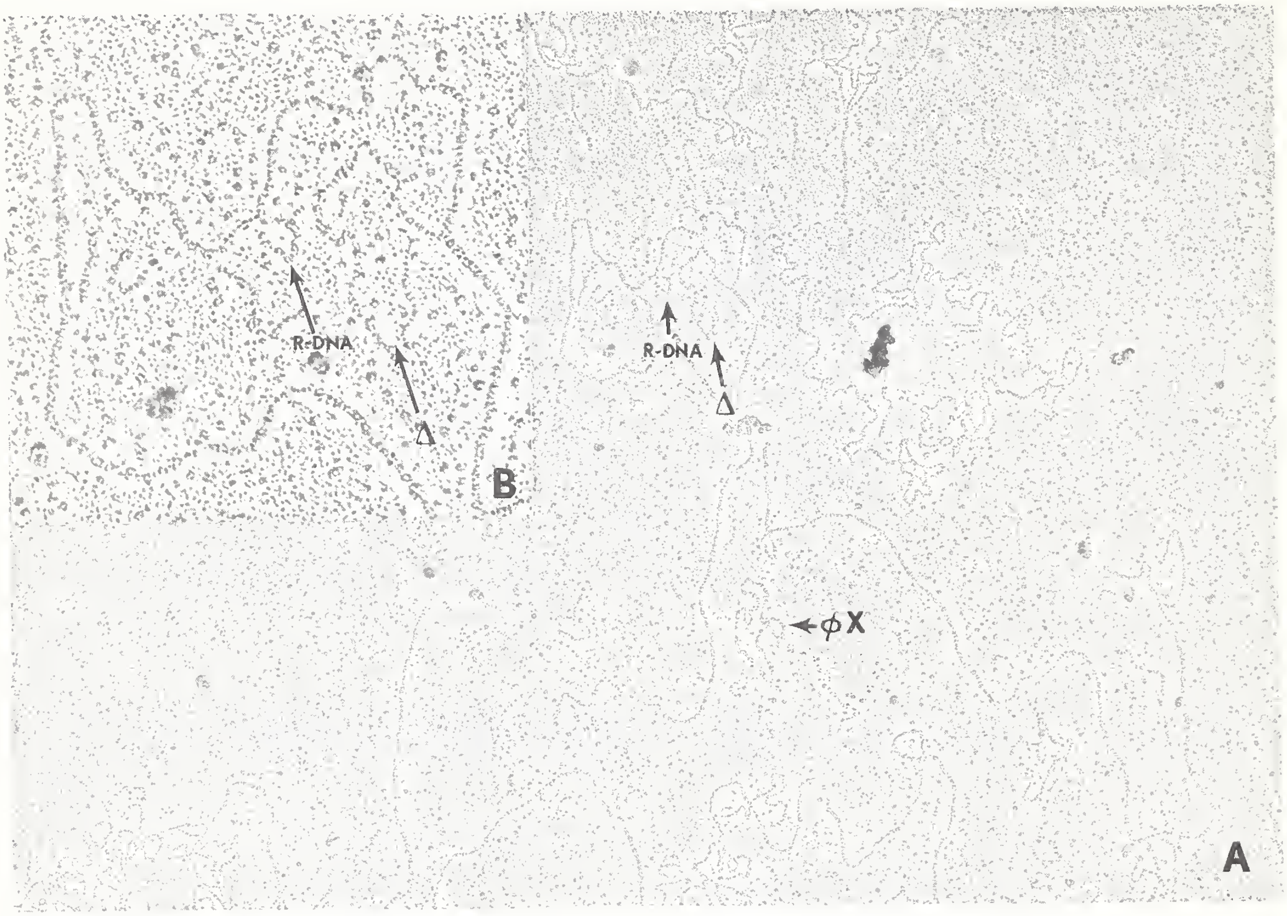


Figure III.

Table 1. SIMILARITY MATRIX FOR TEMPERATURE *B. SUBTILIS* PHAGES

Group		φ105	ρ10	ρ6	ρ14	SP02	φ3T	ρ11	SP16
I	φ105	100(a)							
	ρ10	81 (89)	100						
	ρ6	80 (89)	88 (96)	100					
	ρ14	87 (93)	88 (93)	81 (89)	100				
II	SP02	53 (74)	63 (78)	53 (74)	47 (67)	100			
III	φ3T	18 (44)	26 (48)	16 (41)	21 (44)	29 (63)	100		
	ρ11	17 (44)	25 (44)	16 (41)	21 (44)	24 (52)	100 (100)	100	
IV	SP16	17 (44)	14 (33)	11 (37)	15 (37)	20 (56)	23 (63)	23 (63)	100

(a)Similarity coefficients (as percentage(16)) derived from twenty-seven characters pertaining to serology, host range, immunity and resistance were used to compare temperate phages. Numbers in parenthesis are matching coefficients (as percentage (16)).

TABLE 2. HOST RANGE OF TEMPERATE BACILLUS PHAGES(a)

	Phages								
Hosts	φ105	SP02	φ3T	ρ6	ρ10	ρ11	ρ14	ρ18	SP16
B. subtilis 168	+	+	+	+	+	+	+	+	+
B. subtilis 3610	+	+	+	+	+	+	+	+	+
B. subtilis W23	-	-	-	-	-	-	-	-	+
B. amyloliquefaciens H	-	-	-	-	-	-	-	-	+
B. pumilis	-	-	-	-	-	-	-	-	+
B. licheniformis	-	-	-	-	-	-	-	-	-(b)
B. globigii	+	+	+	-	+	+	+	-	+

(a)Dilutions of a phage lysate (10^{-2}, 10^{-4}, 10^{-6}) were spotted (10 µl) on a lawn of washed spores; + indicates turbid plaques as well as total lysis occur in the spotted zone, - indicates that individual plaques do not form although killing may occur.

(b) Thorne and Merle reported that SP16 plated on *B. licheniformis* 9945A. (Microb. Gen. Bull. 43:7-10).

Actions

The Guidelines require that major actions taken by the Director of NIH on topics relating to recombinant DNA research are to be published in this Bulletin. In the Issues Pending *section, topics which are currently under discussion are briefly described. For example, they may include issues which are being examined by the Office of Recombinant DNA Activities or the NIH Recombinant DNA Advisory Committee. In the* Issues Resolved *section are presented the actions taken by NIH on issues which have been pending. These will be presented in their entirety as printed in the Federal Register or in memos to the Institutional Biosafety Committees.*

Issues Pending

The NIH Recombinant DNA Advisory Committee at its last meeting was requested to advise the NIH on the following issues. The actions taken by NIH will be reported in the next issue of the Bulletin.

1. The cloning of DNA from higher eukaryotes into EK2 systems and the return of the cloned DNA to higher eukaryotes under appropriate physical containment conditions.
2. The cloning, under P1 physical containment, of DNA into invertebrate cells in culture using organelle, plasmid or chromosomal DNA as vectors.
3. The cloning, under P1 physical containment, of DNA into vertebrate cells in culture using organelle, plasmid or chromosomal DNA as vectors.
4. Criteria for approval of lower eukaryotes as HV1 & HV2 systems. Specifically requested for HV1 are laboratory strains of *Saccharomyces cerevisiae* and modified strains of *Neurospora crassa.*
5. A request by Dr. David Botstein, et. al., for approval of a yeast HV2 system based on loss of mating abililty.
6. The use of unmodified *N. crassa* as HV1 systems when higher levels of physical containment are used.
7. New lists of organisms which have been shown to exchange DNA and which, therefore, should be exempt from the Guidelines (when experiments only involve pairs of organisms on these lists). One list involves certain species of *Bacillus* and the other involves certain pairs of *Streptomyces* species.
8. The approval of asporogenic mutant derivatives of *B. subtilis* as HV1 systems using specific plasmids.
9. The use of the P3 level of containment in those cases where two bacterial species do not exchange DNA.
10. The levels of containment for the shotgun cloning of primate and other mammalian DNA can be P3 + EK1 as an alternative to the present P2 + EK2.
11. The cloning in *B. subtilis* under P2 conditions of DNA from *E. coli, S. cerevisiae* and *Streptomyces coelicolor.*
12. The cloning in *Streptomyces coelicolor* under P2 conditions of DNA from *B. subtilis, E. coli,* and *Staphylococcus aureus* plasmids.
13. The use of Ff single-strand bacteriophages (such as M13) as vectors with *E. coli* K-12 for EK1 containment.

"Cosmids" as Vectors under EK1 and EK2 Conditions

The Recombinant DNA Advisory Committee and its Host-Phage Subcommittee recently examined the "cosmid" for use as a cloning vector. Briefly, a cosmid is a hybrid vector composed of a plasmid plus the sticky ends (*cos* sites) of lambda; it can be packaged *in vitro* as a phage but grown in *E. coli* as a plasmid. (Examples of this system and additional information can be obtained from a publication by J. Collins and B. Hohn in the Proc. Nat. Acad. Sci., USA, vol. 75 (1978) 4242-4246).

For use under EK1 conditions, it will be necessary to follow procedures that apply to EK1 host-vector systems (see NIH Guidelines). Also, the cosmid should not contain material which can give rise to conjugal plasmids in the cosmid host.

The Host-Phage Subcommittee has also recommended a set of criteria which will be used for the acceptance of a cosmid system for EK2 host-vector containment. In applying for approval of an EK2 cosmid system the investigators should submit to ORDA the following information:

1. Since the cosmid will persist in its host in the plasmid state, the system would have to pass all tests for a plasmid-host system. Since the cosmid requires a lambda sensitive host, *x*1776 will not be useable. Therefore, whatever new host is selected it will have to meet necessary criteria regarding plasmids and plasmid transfer.

2. Another problem which might be anticipated with the cosmid system would be the release of phage particles carrying the cosmid during the initial packaging or during some later manipulations. Therefore, a limit is placed on any such production of particles of less than 10^9 total particles in any given experiment (i.e., *in vitro* packaging).

3. In addition to transfer of the cloned piece by mobilization of the plasmid, the possibility of transfer by infection of the cosmid-containing strain with lambda and subsequent packaging of the cosmid and its cloned piece must be considered. This might result in infection of wild-type lambda-sensitive strains. The probability of escape via this route is the product of four factors:

a) Survival of the host strain outside the laboratory.

b) The frequency with which the cosmid-carrying strain meets lambdoid phages in the outside environment. This might occur either by infection or Hfr transfer.

c) The frequency with which an escaping lambda particle containing a cosmid plus cloned segment meets a second lambda-sensitive host.

d) The probability of packaging the cosmid and cloned piece after infection of the cosmid-containing host. This last number should be calculated from the following experiment:

A model recombinant carrying a detectable marker of a size appropriate for packaging by lambda should be constructed and grown in the proposed EK2 host. This strain should be infected with lambda at an moi < 10, and the resulting phage lysate used to infect a lambda lysogen. These infected lysogens, after appropriate incubation for expression of the cloned marker, should be plated to determine the number of cells which have received the cloned marker.

The results of this experiment can be expressed as:

$$\frac{\text{\# of cloned markers transferred to new cells (= output)}}{\text{\# of cloned markers in original strain (= input)}}$$

An appropriate positive control to demonstrate the ability to recover the cloned marker after infection of the recipient cells should also be performed.

Data on (a) will be submitted as part of the necessary testing of the plasmid-host. An estimate of (c) has been made previously, of approximately $<10^{-3}$. (d) should be experimentally determined for the cosmid proposed as EK2 vector. (b) has not been previously estimated, but data on the density of lambdoid phages in sewage or other environments could be used for such an estimate. The product of these four factors should be less than $1/10^8$.

Male Specific Bacteriophages and EK1 Containment

The M13, fd, and other related single-strand bacteriophages in conjunction with *E. coli* K-12 hosts have been examined for their suitability as EK1 systems. Since these bacteriophages are male-specific, they are not presently approvable for use with their natural hosts as the Guidelines do not allow EK1 hosts that contain conjugation-proficient plasmids.

However, these bacteriophages in conjunction with *E. coli* K-12 strains that do not contain conjugation-proficient plasmids (i.e., F^-) are acceptable for experiments that require EK1 biological containment. Since the host strains are not the natural hosts, the means of infection would involve transfection. Procedures for transfection are similar to those for transformation of *E. coli*. Because the phages are exuded into the medium without cell lysis, large yields can be obtained.

In addition based on the recommendations of the NIH Recombinant DNA Advisory Committee (RAC) and its bacteriophage subcommittee, these bacteriophages are now also approved for use with bacteria that contain conjugation-defective plasmids:

Conjugation-deficient mutants, such as the *tra*D and *tra*I mutants of the F factor may be used with Ff bacteriophages if the mutants have been shown to exhibit low levels of transfer (of the order of 10^{-5} or less) and also have low reversion rates (such as found for deletion or double-mutants).

This action is based on the intent of the Guidelines; that is, the hosts shall not contain conjugation-proficient plasmids. These restrictions on the transfer defective mutants combined with the natural containment features of these bacteriophages in their hosts provide more than adequate EK1 containment.

The use of these or any bacteriophages in the presense of conjugationally-proficient plasmids has *not* been approved for EK1 containment. This requires further examination by the RAC and will be discussed at the next meeting of the RAC as part of a general proposal to allow such use.

[A complete report of the use of the single-strand male-specific bacteriophages as vectors in EK1 systems is available from the Office of Recombinant DNA Activities.]

dA-dT Tailing with Certified Vectors

The dA-dT tailing method can be used to enable the joining of DNA fragments of EK2 vectors. The use of this method is considered to lead only to a minor modification of a certified vector.

χ2282 in EK2 Experiments

The *E. coli* K-12 strain χ2282 has been given approval for limited use as an EK2 host. This ThyA+ variant of χ1776 may only be used for EK2 containment in experiments involving the cloning and expression of the dihydrofolate reductase gene. χ2282 is not certified for general use in EK2 systems.

Data to be Submitted for the Certification of EK2 Host-Plasmid Systems

The Host-plasmid Subcommittee of the RAC recently reevaluated the criteria for acceptance of EK2 systems. Many of the following instructions to investigators concerning data to be submitted, were described in an earlier Bulletin (Vol. 1, No. 2, Winter, 1978, p. 5 see II, B). The major changes include shorter matings (see B), testing an additional plasmid (see II, C), and the absence of triparental matings.

I. *Survival*

(A) Time-course experiments must show that survival of the host-plasmid falls to 10^{-8} or lower within 24 hours of incubation *in vitro* under nonpermissive conditions (i.e., conditions that have been demonstrated to obtain in the mammalian intestinal tract).

(B) The above loss of survival must depend on at least two phenotypic traits which contribute independently to the overall containment (i.e., either one alone must be demonstrated to produce 10^{-8} survival within 24 hours under non-permissive conditions). At least one trait must be non-revertible, and the reversion rate of the others must be shown to be equal or less than 1×10^{-8} per cell per generation.

II. *Transmission*

Although only non-conjugative plasmids may be used in EK2 systems, the flora of the intestinal tract include bacteria that harbor conjugative plasmids; transfer of the latter to an EK2 cell may result in the mobilization of the EK2 plasmid* for transfer to a third, recipient bacterium.

The probability of this occurring will be the product of four independent probabilities:

(1) The probability (P_1) that the EK2 cell will encounter a bacterium that harbors a mobilizing plasmid and is capable of conjugating under the prevailing environmental conditions.

(2) The probability that the mobilizing plasmid will be transferred to the EK2 cell. This can be measured experimentally under optimal mating conditions *in vitro*, and expressed as the transfer rate, R_1.

(3) The probability (P_2) that an EK2 cell which has received a mobilizing plasmid will encounter a competent recipient bacterium, and conjugate with it under prevailing environmental conditions.

(4) The probability that the EK2 plasmid will be mobilized and transferred to the recipient. This can be measured under optimal mating conditions *in vitro*, and expressed as the transfer rate, R_2.

In actual experiments† on transfer between *E. coli* strains *in vivo* using host-plasmids for which R_1 and R_2 are both very high ($> 10^{-2}$), the observed transfer frequencies (roughly corresponding to $P_1 \times R_1$, and $P_2 \times R_2$, respectively) were less than 1×10^{-9}, suggesting that P_1 and P_2 are each less than 1×10^{-7}. Since, however, much higher values might obtain under other conditions (e.g., in sewage, or in animals fed antibiotics), it would seem prudent to demand that a significant degree of containment be contributed by the factors R_1 and R_2.

A maximal value of 1×10^{-6} for the product $R_1 \times R_2$, for example, would give a figure of less than 1×10^{-8} for the overall rate of transfer ($P_1 \times R_1 \times P_2 \times R_2$), even if $P_1 \times P_2$ were as high as 1×10^{-2}, rather than the observed value of less than 1×10^{-14}.

In view of these considerations, the investigator is required to furnish data from the following types of experiments:

(A) The rate of transfer (R_1) of each of several mobilizing plasmids (see below) from their respective donors to the proposed EK2 system must be measured under optimal, permissive conditions, with 90-minute matings *in vitro*.

(B) The rate of transfer (R_2) of the EK2 plasmid from the EK2 system harboring each mobilizing plasmid must be measured under optimal, permissive conditions with matings of at least three hour duration.

(C) The above tests must be done with one mobilizing plasmid from each of the following compatibility groups: FII, N, I, and P. A genetically derepressed plasmid should be used in each case, if available.

(D) The product, $R_1 \times R_2$, must be equal to or less than 1×10^{-6} in the case of derepressed mobilizing plasmids, and must be equal to or less than 1×10^{-8} in the case of repressed plasmids.

(E) For each mobilizing plasmid, a positive control must be carried out under permissive conditions to confirm the efficacy of the test. In these control experiments, the donor-mobilizing plasmid system and the recipient bacterial strain must be the same as used in the above experiments. In these matings, $R_1 \times R_2$ (separately measured) must be equal to or greater than 1×10^{-3} in the case of derepressed plasmids and must be equal to or greater than 1×10^{-5} for repressed plasmids.

(F) Matings should be carried out at initial cell concentrations of 10^8 cells/ml and a donor to recipient ratio of 1. Survival of the EK2 and control host during the three-hour mating period must be measured, and the transfer rates must be calculated in terms of the number of viable EK2 cells at the end of the mating period.

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*We will refer to the separate components of the EK2 system as the "EK2 plasmid" and the "EK2 cell".

†S. Falkow, personal communication.

News And Comment

The New RAC

The number of people on the NIH Recombinant DNA Advisory Committee (RAC) has been increased to twenty-five. The following is a list of the committee members and the date of expiration of their term of appointment (in parenthesis):

Chairman
Setlow, Jane K, Ph.D. .... (80)
Biologist, Brookhaven National Laboratory
Upton, Long Island, New York 11973

Ahmed, Abdul Karim, Ph.D. .... (82)
Senior Staff Scientist
National Resources Defense Council
New York, New York 10017

Baltimore, David, Ph.D. .... (82)
Professor of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Broadbent, Francis, Ph.D. .... (80)
Professor of Soil Microbiology
Department of Land, Air
and Water Resources
University of California
Davis, California 95616

Campbell, Allan M., Ph.D. .... (81)
Professor, Department of Biology
Stanford University
Stanford, California 94305

Cason, Zelma .... (81)
Supervisor of Cytopathology
Laboratory
University of Mississippi Medical
Center
Jackson, Mississippi 39216

Day, Peter, Ph.D. .... (80)
Chief, Division of Genetics
Connecticut Agricultural
Experiment Station
New Haven, Connecticut 06504

Goldstein, Richard, Ph.D. .... (82)
Assistant Professor of Microbiology
and Molecular Genetics
Harvard Medical School
Boston, Massachusetts 02115

Gottesman, Susan, Ph.D. .... (81)
Senior Investigator
Laboratory of Molecular Biology
National Cancer Institute
Bethesda, Maryland 20014

Hornick, Richard A., Ph.D. .... (81)
Chairman, Department of Medicine
University of Rochester
School of Medicine
Rochester, New York 14642

King, Patricia, J.D. .... (82)
Professor of Law
Georgetown University Law Center
Washington, D.C. 20001

Krimsky, Sheldon, Ph.D. .... (81)
Acting Director, Program in Urban Social and
Environmental Policy
Tufts University
Medford, Massachusetts 02155

Kutter, Elizabeth, Ph.D. .... (79)
c/o Department of Nutrition
University of California
Davis, California 75616

Novick, Richard, M.D. .... (81)
Chairman of Plasmid Biology
Public Health Research Institute
New York, New York 10016

Parkinson, David, B.M., B.Ch. .... (81)
Associate Professor of Occupational
Health
University of Pittsburgh
Pittsburgh, Pennsylvania 15261

Pinon, Ramon, Ph.D. .... (80)
Assistant Professor of Biology
B-022 Bonner Hall
University of California
San Diego, California 92093

Proctor, Samuel, Ph.D. .... (80)
Professor of Education
Rutgers University
New Brunswick, New Jersey 08903

Redford, Emmette, Ph.D. LL.D. .... (79)
Ashbel Smith Professor of Government
and Public Affairs
Lyndon B. Johnson School of Public Affairs
University of Texas at Austin
Austin, Texas 78712

Rowe, Wallace, P., Ph.D. .... (79)
Chief, Laboratory of Viral Diseases
National Institute of Allergy &
Infectious Diseases
National Institutes of Health
Bethesda, Maryland 20014

Spizizen, John, Ph.D. .... (79)
Member and Chairman Department of
Microbiology
Scripps Clinic & Research Foundation
La Jolla, California 92093

Thornton, Ray, J.D. .... (82)
P.O. Box 521
Sheridan, Arkansas 72150

Walters, LeRoy, Ph.D. .... (80)
Director, Center for Bioethics
Kennedy Institute
Georgetown University
Washington, D.C. 20057

Williams, Luther, Ph.D. .... (81)
Associate Professor of Biology
and Assistant Provost
Department of Biological Sciences
Purdue University
West Lafayette, Indiana 47907

Young, Frank, M.D., Ph.D. .... (80)
Professor and Chairman
Department of Microbiology
University of Rochester
Rochester, New York 14642

Zaitlin, Milton, Ph.D. .... (80)
Professor, Department of Plant Pathology
Cornell University
Ithaca, New York 14853

Executive Secretary

Gartland, William J., Jr., Ph.D.
Director, Office of Recombinant
DNA Activities
National Institute of Health
Bethesda, Maryland 20014

COGENE

The Committee on Genetic Experimentation (COGENE) was created in 1977 by the General Assembly of the International Council of Scientific Unions (ICSU). The ICSU is an international nongovernmental scientific organization of 18 autonomous international Scientific Unions and more than 60 National Members, i.e. academies of science, research councils or similar scientific institutions.

The COGENE is a committee similar to others formed by ICSU whenever a task is defined that is of major interest to several unions and the program is of a long-term nature. COGENE is supported by seven member unions - Pure and Applied Chemistry, Biological Sciences, Biochemistry, Pure and Applied Biophysics, Nutritional Sciences, Pharmacology and the Immunological Societies - and includes a number of scientists prominent in the field of recombinant DNA research, which is currently the major focus of interests of COGENE and the reason why the Committee came into being.

Its purposes and objectives have been defined as follows:

COGENE is established to serve as a nongovernmental, interdisciplinary and international council of scientists and as a source of advice for the benefit of governments, inter-governmental agencies, scientific groups, and individuals concerning recombinant DNA activities.

Among its purposes shall be:

a) to review, evaluate and make available information on the practical and scientific benefits, safeguards, containment facilities and other technical matters,

b) to consider environmental, health-related and other consequences of any disposal of biological agents constructed by recombinant DNA techniques,

c) to foster opportunities for training and international exchange, and

d) to provide a forum through which interested national, regional and other international bodies may communicate.

COGENE shall also consider, if necessary, other related activities which may give rise to public concern.

(Additional information concerning COGENE can be obtained from Professor W.J. Whelan, Department of Biochemistry, University of Miami School of Medicine, Miami, Florida 33152).

COGENE Reports

The following are summaries of three reports emanating from working groups of COGENE. The full reports can be obtained from Dr. W.J. Whelan (see above).

Risk Assessment

A.M. Skalka (USA) *convenor*
G. Bernardi (France)
V. Sgaramella (Italy)

The Working Group has been charged with the task of gathering information related to assessment of risks associated with recombinant DNA research. The information in this report was obtained by observation and participation at a number of meetings and hearings, by survey of the literature and by direct inquiry to national and international organizations, and individual scientists.

Our analyses indicate that the concerns regarding recombinant DNA research can be summarized as follows:

1. Organisms carrying recombinant DNA may spread in the natural environment and disrupt existing ecological equilibria.
2. These organisms might produce some toxic or noxious substance, or otherwise cause disease.
3. By exploiting this technology, scientists may be crossing some hypothetical barrier to DNA exchange between eukaryotes and prokaryotes

and thus affect pathogenicity or dispersion of pathological agents.

Reports from the Falmouth Meeting (section IV) and COGENE-sponsored analyses of *E. coli* K-12 systems (section VI) show that many relevant experiments have already been conducted by scientists in such closely related fields as epidemiology and infectious diseases. Their observations that *E. coli* K-12 has a limited prospect of survival and their consensus that this bacterium cannot be converted into an epidemic pathogen by laboratory manipulations with DNA inserts, do much to satisfy the first concern mentioned above. In addition (section VII), long-term monitoring of laboratory workers who routinely handled K-12 organisms carrying transmission-proficient plasmids showed no bowel colonization despite the fact that the work was carried out without any special precautions. If the organisms which carry recombinant DNA cannot spread in the natural environment, then clearly the other concerns are also diminished. The conclusion of a NIH/EMBO Virology Workshop (section V) was that recombinant organisms carrying viral inserts cannot be more hazardous than the viruses themselves, and in some instances may provide an opportunity to work more safely with virulent agents.

As can be seen in the report, important information on the nature and possible consequences of the manipulations used in recombinant DNA technology comes from experiments in various fields. For example, attempts to understand the biological role of restriction enzymes have revealed that prokaryotic and eukaryotic DNAs can recombine *in vivo*. Other studies, including those with *Agrobacterium tumefaciens* and plant cells, also show that in some cases there is no natural barrier to exchange and expression of genes across the hypothetical prokaryotic-eukaryotic barrier. On the other hand, studies of animal virus genomes and eukaryotic genes for differentiated functions have revealed previously unsuspected possible barriers to their expression in prokaryotic backgrounds. It is clear that further studies of the molecular genetics, pathogenicity, ecology and other properties of organisms will continue to provide information relevant to the assessment of risk and that this source is no less important than specifically targeted experiments.

Replies to our Questionnaire (sections I and II) indicate that there are several projects currently underway directed specifically towards risk assessment. These include the two separate polyoma virus experiments currently sponsored by EMBO and by NIH and several NIH contracts aimed at testing and verification of EK2 and EK3 systems. Preliminary results, as well as conclusions from the NIH-EMBO Virology Workshop indicate that these studies are unlikely to reveal unknown hazards. These and other experiments which are aimed at elucidating various aspects of the ecology and natural history of microorganisms should provide useful information and will be important in the development of host-vector systems other than those based on *E. coli* K-12.

In summary, our analyses have revealed no scientific findings to justify any of the three concerns listed above: no risk unique to recombinant DNA research has been identified. Available evidence indicates that recombinations of the type made possible by this new technology can occur in Nature. Evaluation of *E. coli* K-12 shows that this bacterium is essentially harmless and that insertions of segments of foreign DNA into its genome cannot alter this property. With few exceptions, it seems likely that the same will prove true of other host bacteria. These conclusions cannot be ignored if the guidelines governing recombinant DNA research are to remain rational and useful.

Recombinant DNA Guidelines Report

S.N. Cohen (USA), covenor
G. Ada (Australia)
A.A. Bayev (USSR)
G. Bernardi (France)
L. Bogorad (USA)
P. Starlinger (FRG)
J. Tooze (E.M.B.O.)

The Working Group of Recombinant DNA Guidelines was established by COGENE in May 1977 and charged with the responsibility of 1) obtaining information about the status and content of Recombinant DNA Guidelines in different nations and 2) analyzing, comparing and evaluating the provisions of the various national guidelines and the premises on which these provisions are based. Shortly after its formation the Working Group prepared and circulated a short questionnaire to the scientific liason for each nation represented in the International Council of Scientific Unions. A second questionnaire was distributed to the appropriate scientific representative of each nation which had indicated that recombinant DNA experimentation and/or the preparation of guidelines governing this research was underway.

By the time of the meeting of the Working Group in Paris on April 7-8, 1978, completed questionnaires and/or copies of the national guidelines had been received from 49 countries having scientific societies affiliated with ICSU and the information provided had been tabulated by computer. The accumulated questionnaire data and the results of the Working Group's analysis and evaluation of the guidelines documents themselves form the basis for this interim report.

At the end of March 1978, at least 367 recombinant DNA projects were estimated to be underway in 155 laboratories in 15 countries. Seven of these were at a high level of containment, 76 were at a moderate level, and the remainder at a level of containment designated by the respondent as "low". Seventeen nations had drawn up guidelines for recombinant DNA experimentation, and these were in force in 13 nations. Five of the nations had prepared their own guidelines, whereas the rest had adopted or modified guidelines of the United States or the United Kingdom. Of the responding nations that had not drafted guidelines by the time of the response, 13 intended to establish guidelines, while 4 indicated no intention to establish guidelines. Sixteen recombinant DNA projects were indicated as being underway where no guidelines existed.

The guidelines of 4 nations were reported as being entirely voluntary. Eight nations have guidelines that are enforceable through a research funding mechanism, and 2 nations have legally enforceable guidelines. The mechanism for authorizing research varies in different nations: in some nations authorization is provided to institutions, whereas in others it is granted to individual laboratories and/or scientists. Six nations require specific training for researchers in this field, and 7 offer training courses. Although the guidelines of 8 nations indicate that the precautions required for recombinant DNA experimentation can be modified on the basis of experience in this area of research, only 4 nations specify any criteria or mechanism for such modification.

A full tabulation of information obtained from completed questionnaires will be included with the next report of the Working Group.

The Working Group's analysis of the various national guidelines and of the questionnaire responses has led to the following preliminary conclusions:

(1) During the past several years, guidelines for the conduct of recombinant DNA experiments have been prepared by a number of nations whose national scientific bodies are members of ICSU. Some nations carrying out recombinant DNA experimentation have not promulgated formal guidelines, and some do not inted to do so.

(2) Guidelines of most nations have been derived directly or indirectly from those of the United States, which were the first to be promulgated, and the assumptions implicit or explicit in the United States guidelines have been incorporated in guidelines of other nations. Although fine gradations of risk and containment are specified in most national guidelines, the basis for such distinctions appears to be entirely conjectural. No documentation to support the assumption of risk is provided in any of the guidelines examined.

(3) While the guidelines of other nations have been generally patterned after those of the U.S., there are substantial differences in the various national guidelines. Some have the force of regulations, while others are considered simply as standard operating procedures, and their observance is voluntary. Some guidelines are concerned only with organisms containing recombinant DNA molecules while others also pertain to the recombinant DNA itself. Some have employed an encyclopedic approach which attempts to anticipate and specify conditions for all possible forms of recombinant DNA experimentation, while others have adopted an approach that utilizes a case-by-case analysis for determining the containment necessary for a particular kind of experiment.

(4) Identical experiments require strikingly different levels of precautions in different nations. Moreover, although the general approach to biological and physical containment first proposed in the U.S. guidelines has been adopted by most other nations, the specific characteristics of each containment level differ markedly among different nations.

(5) In some nations, the guidelines specify the maximum level of precaution required, and a national committee may reduce precautions from this level under certain circumstances. In other nations, the guidelines specify the minimum level of precaution required, and precautions may be increased by the actions of local or sub-national bodies.

(6) Those guidelines that have been prepared more recently are in general less restrictive than are the earlier guidelines. This appears to reflect a changing assessment of the risk of recombinant DNA experiments as greater experience in this area of research has accumulated. However, the guidelines of most nations do not have a time lim-

itation clause, and the frequency of revision of guidelines is usually not specified.

(7) The various national guidelines appear to be intended to deal with the risk of a major epidemic, although the chance of initiating an epidemic by recombinant DNA experimentation in *E. coli* K-12 or analogous non-pathogenic organisms appears negligible.

(8) The level of containment required for work with recombinant DNA from known pathogens is often greater than is specified for work with the intact pathogenic organism. The guidelines of some nations effectively prohibit recombinant DNA work with genes from medically important disease-producing organisms, although recombinant DNA methods appear to provide a means of carrying out such studies at less risk than is involved in work with the pathogens themselves.

The full report of the working group will include a history of the efforts to develop guidelines for recombinant DNA experimentation, a discussion, analysis and evaluation of the explicit and implicit assumptions on which the guidelines are based, a comparison of the levels of containment required in various nations for sample experiments, an updated tabular compilation of information provided in complete questionnaires, a statement summarizing th principal findings of the survey, and a section that will present the general conclusions of the Working Group.

The status of guidelines for recombinant DNA experimentation is undergoing rapid change at this time. This appears to be the result of a changing assessment of the conjectural risks that led to the initial formation of guidelines and regulations for research in this area, and the realization of substantial scientific benefits from the use of this research tool.

The information being accumulated by the Working Group on Recombinant DNA Guidelines should be of value to individual scientists as well as to national advisory committees, and should be useful in the formation of any policy statement on this subject by COGENE.

Benefits and Applications of Recombinant DNA Technology

In addition to three members of COGENE, 40 representatives of industry, science and government from seven countries (Denmark, Germany, Japan, Switzerland, UK, USA, USSR) were present.

The consensus of this group was 1) that recombinant DNA technology offers enormous benefits for the development on an industrial scale of new or improved sources of medicines, foods and industrial chemicals, 2) that by the time a micro-organism has been developed for use in industrial fermentation it will have been thoroughly characterized both with regard to the biohazards it may present and the products it will produce and appropriate safety measures will have been devised, 3) that the genetically engineered micro-organisms that will have to be constructed to realize these benefits will not pose any special biohazards that cannot be obtained by the sorts of safety measures already used in the industrial fermentation industry, 4) that although there are no forseeable, insuperable scientific obstacles to prevent the progressive exploitation of the new technology on an industrial scale, several unanswered questions about the molecular biology of micro-organisms and higher organisms must be answered by basic research if the industrial applications are to proceed at an optimal rate, 5) that since the common bacterium *E. coli* is not ideal for industrial fermentation, a range of alternative micro-organisms more suited to the needs of the industry, should be developed as safe hosts for recombinant DNA molecules. Until these alternative host-vector systems are developed the extent to which the potentials of this new biotechnology can be realized will be severely restricted; 6) that industry will abide by national guidelines relating to recombinant DNA research but is also concerned to protect its proprietary rights and requests that procedures be developed to accommodate these two, sometimes conflicting interests; 7) that patent laws relating to the patenting of strains of micro-organisms *per se* and novel gene combinations can be clarified and that COGENE and other agencies help disseminate literature explaining the new DNA technology to those responsible for the execution of patent law.

Technical Information

Detailed instructions concerning data to be submitted for certification can be obtained from the Office of Recombinant DNA Activities, NIH, Bethesda, Md. 20205. Information on tests for EK2 phage certification were presented in Volume 1 Number 1 and for EK2 plasmid certification in this issue.

Certification of a given system does not extend to modifications of either the host or vector component. All modified systems are subject to independent confirmation and approval. The procedures for certification are presented in the Guidelines (Federal Register, December 22, 1978, pp. 60108-60131). Presented in the following tables are the certified EK2 host-vector systems.

Plasmid Systems

Vector	Host	Date Certified	Literature References
pSC101	x1776	12-6-1976	1,2
pCR1*	x1776	(decertified 8-21-78)	1,3,4
pMB9	x1776	4-18-1977	1,5,6,7
pBR313	x1776	7-7-1977	1,7
pBR322	x1776	7-7-1977	1,7

The four host-plasmid systems that have been certified for use as EK2 host-vector systems are listed in the above Table.

*Since the time of certification of the systems employing pSC101 and pCR1, a new set of criteria for EK2 certification has been approved by the Recombinant DNA Molecule Program Advisory Committee. These criteria have been met by the systems employing the plasmids pMB9, pBR313, and pBR322. The Committee recommended the decertification of x1776 (pCR1) as an EK2 host-vector system on the grounds it is inferior with regard to transmissability and further recommended that this system not be allowed for any new cloning since superior plasmids are available. It is therefore the decision of NIH that x1776 (pCR1) is decertified as an EK2 host-vector system. However, existing clones need not be destroyed.

Bacteriophage Systems

Vector	Host	Date Certified	Literature References
λgt*WES* · λB′	DP50*supF*	2-22-1977	8,9
λgt*WES* · λB*	DP50*supF*	6-22-1977	12
λgt*ZJvir* · λB′	*E. coli* K-12	2-22-1977	10
Charon 3A Charon 4A Charon 16A	DP50 or DP50*supF*	3-1-1977	11
Charon 21A	DP50*supF*	8-12-1978	13

In Volume 1, Number 1 of the Bulletin, Charon 16A was identified as Charon 316A. The designation Charon 16A is currently in use.

In EK2 systems, recombinant molecules may be introduced into propagation hosts either by transfection with naked DNA or by infection with viral particles produced by in vitro packaging. When in vitro packaging is used:

(1) The packing extract must be free from viable bacteria.

(2) Any packaging protocol may be used, provided that control experiments on the packaging of EK2 vector DNA meet one of the following two criteria:

(a) The number of amber+ phages produced must be less than 10^{-6} times the number of amber- phages. If shotgun populations are to be propagated in bulk culture or by confluent lysis methods, this measurement must be made on packaged EK2 vector DNA propagated to the same extent.

(b) If the total number of amber- phages produced in a packaging experiment is less than 10^{6}, and if the shotgun population is not to be propagated in bulk, the number of observed amber plaques must be zero.

(3) The above tests must be done on each batch of packaging extract used.

(4) Any individual clone isolated from the shotgun must be tested for retention of the safety characteristics of the vector before it can be used for bulk propagation.

(5) A description of the packaging protocol should be filed with NIH for information, but NIH approval is not required provided that the above numerical criteria are met.

Certain types of small-scale experiments are allowed in hosts other than DP50*supF*. Specifically, transfection and very small scale experiments (i.e., less than 1.0 ml, plating for single plaques for screening purposes) are allowed in other *E. coli* K-12 hosts. However, plate lysates or other procedures (for instance, growth of small lysates in liquid) which would yield large numbers of phage (10^{9} or greater) are to be done only in the DP50*supF* host.

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Characterized Clones

The enclosed table is an addendum to the previously published tables of characterized clones. Data on characterized clones were submitted by individual investigators to the Office of Recombinant DNA Activities (ORDA). These requests were individually reviewed by the NIH Recombinant DNA Advisory Committee. Committee actions to permit reduction of containment levels were based on evidence that clones are rigorously characterized (e.g., hybridization and restriction endonuclease fragmentation analysis nucleotide sequencing, R-loop analysis, and), free of harmful genes (e.g., sequences contained in indigenous tumor viruses or which code for toxic substances), the biological properties of the vector are maintained, and the cloned fragment is genetically stable.

In accord with the 1978 Guidelines, requests to lower containment levels for characterized recombinant clones by one step in physical or biological containment should be submitted to the Institutional Biosafety Committee (IBC), which can approve such reductions. The reduction of containment levels for characterized clones derived from primate DNA require NIH review and approval as does the reduction for more than one step in containment. These requests should be submitted to ORDA.

REDUCTION OF CONTAINMENT LEVELS OF CHARACTERIZED RECOMBINANT DNA CLONES BY NIH RECOMBINANT DNA ADVISORY COMMITTEE

CLONE NUMBER	ORIGIN OF CLONE	CONTAINMENT APPROVAL FOR CHARACTERIZED CLONE	DATE APPROVED	BASIS OF CHARACTERIZATION	LITERATURE REFERENCES
K149	Mouse Ig cDNA kappa light chain	P2 + EK1	8-10-78	In situ hybridization, R-loop analysis, sequencing	1
K41	Mouse Ig cDNA kappa light chain	P2 + EK1	8-10-78	In situ hybridization, R-loop analysis, sequencing	2
MβG2	Mouse beta globin maj genomic	P3 + EK1/P2 + EK2	8-10-78	In situ hybridization, restriction site analysis, R-loop analysis	3
MβG3	Mouse beta globin min genomic	P3 + EK1/P2 + EK2	8-10-78	"	4
MK2	Mouse Ig kappa light chain genomic	P3 + EK1/P2 + EK2	8-10-78	"	5
MK3	Mouse Ig kappa light chain genomic	P3 + EK1/P2 + EK2	8-10-78	"	6
Mα1	Mouse genomic alpha globin	P3 + EK1/P2 + EK2	8-10-78	"	7
POvE12	Chicken ovalbumin	P2 + EK1	8-9-78	Restriction analysis, hybridization	8
PAFP5	Rat alpha fetoprotein	P2 + EK1	8-9-78	Hybridization, gel analysis, restriction analysis	9, 10
pCg13 pCg45	Chicken procollagen	P2 + EK1	9-19-78	Restriction analysis, hybridization, sequencing	11
pλ2b(11)7 pκ(11)24	Mouse Ig heavy chain Mouse Ig light chain	P2 + EK1	8-9-78	Restriction analysis, hybridization	12
pα(558)13 pμ(3741)9	Mouse Ig heavy chain Mouse Ig light chain	P2 + EK1	8-9-78	Restriction analysis	13
pHα1 pHβG1 pHγG1	Human cDNA globin	P2 + EK1	10-12-78	Hybridization, sequence [partial] analysis	14
3Hb117	Mouse globin	P2 + EK1	10-27-78	Hybridization, sequencing	15
PXlvc 2,8, 10-13, 18-24, 27-30, 37,72, 73	Xenopus laevis vitellogenin cDNA	P2 + EK1	12-12-78	R-loop analysis, hybridization purity of starting material	16, 17
pHb1008	Chicken alpha globin cDNA	P2 + EK1	12-18-78	Restriction mapping, sequence analysis,	18
pH21-1	Mouse Ig heavy chain cDNA	P2 + EK1	12-18-78	Restriction analysis, nucleotide sequence analysis	19
pSβ2B pSβC69 pSα56	Sheep globin cDNA	P2 + EK1	12-19-78	Hybridization, restriction analysis, purity of starting material	20, 21

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Announcements

International Conference On Recombinant DNA

A conference is to be held at Wye Agricultural College, United Kingdom from April 1 to 4 which will be sponsored by the Royal Society and COGENE (see page). The purpose of the conference is to review the current status of recombinant DNA research and technology and discuss issues of research support, regulation, and practical applications. The meeting will be divided into the following sections:

1. Recombinant DNA techniques put into biological perspective
2. Achievements of recombinant DNA research
3. Practical benefits of recombinant DNA research
4. Re-examination of basic assumptions

The proceedings of this meeting are to be published by Pergamon Press (edited by Dr. Joan Morgan and Dr. W.J. Whelan) by approximately August, 1979.

Laboratory Safety Training Short Courses

Laboratory safety training short courses for 1979 are set as conducted by the University of Minnesota School of Public Health under contract from the National Cancer Institute Office of Research Safety.

Courses on "Biohazard Containment and Control for Recombinant DNA Molecules" will be presented May 30-31, University of California at Los Angeles; June 12-13, University of Wisconsin, Madison and June 26-27, National Institutes of Health, Bethesda, MD. These courses are directed at principal investigators, scientists, and senior technicians working with genetic recombinants. Lectures and workshops will emphasize interpretation of the N.I.H. Guidelines for Recombinant DNA Research.

Two courses on "Biohazard and Injury Control in the Biomedical Laboratory" will be held. They are set for September 11-13, Ohio State University, Columbus, and December 11-13, National Institutes of Health, Bethesda. Emphasis at these courses is placed on biohazards and technicians working in fields related to cancer virology.

Support from the National Cancer Institute enables the University of Minnesota to offer both courses tuition free. Participants pay only for travel and living expenses. Enrollment is limited and early application is recommended.

Information on the courses can be obtained from Donald Vesley, Ph.D., Professor, School of Public Health, 1158 Mayo Memorial Building, 420 Delaware St. S.E., University of Minnesota, Minneapolis, MN 55455. Telephone (612) 373-5943.

Future Meetings of the NIH Recombinant DNA Advisory Committee

May 21-23, 1979
September 5-7, 1979
December 5-7, 1979

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Each issue of the Bulletin contains a set of citations to articles published in the scientific literature and concerned with aspects of recombinant DNA technology. The bibliographic citation list is generated from the previous three monthly issues of Index Medicus by Medlars II, the national interactive retrieval service of the National Library of Medicine. There is a lag of several months between publication of an article and its appearance in the Index Medicus. Only those journals listed in the Index serve as citation sources.

We acknowledge with gratitude the help and cooperation of the staff of the Medical Subject Headings Branch of the National Library of Medicine.

Subscribers are encouraged to submit citations to articles they believe are relevant to recombinant DNA activities.

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